Chapter 5

Astrocytes in Pathogenesis of ALS Disease and Potential Translation into Clinic

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Abstract

Astrocytes are the major cell population in the central nervous system (CNS) and play pivotal role in CNS homeostasis and functionality. Malfunction of astrocytes were implicated in multiple neurodegenerative diseases and disorders, including amyotrophic lateral sclerosis (ALS), spinal cord injury (SCI), brain stroke, Parkinson's disease (PD), and Alzheimer disease (AD). These new insights led to the rationale that transplantation of healthy and functional human astrocytes could support survival of neurons and be of therapeutic value in treating neurodegenerative diseases. Here, we will mainly focus on the role of astrocytes in ALS disease, the major cell sources for generation of human astrocytes, or astrocyte like cells and show how multiple preclinical studies demonstrate the efficacy of these cells in animal models. In addition, we will cover immerging early stage clinical trials that are currently being conducted using human astrocytes or human astrocyte like cell population.

Keywords: astrocytes, amyotrophic lateral sclerosis (ALS), hSOD1^{G93A}, neurodegenerative diseases, mesenchymal stem cells (MSCs), glial cells

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease that affects upper and lower motor neurons (MN) in the brain and spinal cord, respectively, this leads to paralysis and eventually death, mainly due to respiratory failure [1, 2]. The disease is incurable and fatal within 3–5 years of first symptoms. About 90% of ALS cases are sporadic, and about 10% of ALS cases are familial (with genetic background). Around 45% of familial ALS cases are caused by hexanucleotide expansion in the C9orf72 gene [3], while approximately 20% of the cases are associated with mutations in the Cu/Zu superoxide dismutase

(SOD1) gene [4]. Other mutations consist of RNA/DNA binding proteins FUS, TAR DNA binding protein-43 [5, 6]. hSOD1^{G93A} high copy number transgenic rats and mice recapitulate the disease phenotype and serve as an animal model for ALS [7, 8].

In ALS, the motor neuron degeneration process is accompanied by signs of oxidative stress and mitochondrial dysfunction, inclusion bodies, impairment of RNA processing, neurofilament aggregation, loss of axonal transport, disruption of the neuromuscular junction, and axon demyelination [9]. In the extracellular environment, there are signs of toxicity, resulting from glutamate accumulation, neuroinflammation, and blood barrier disruption. Astrocytes are the most abundant cells in the central nervous system (CNS) and are necessary for the protection and regeneration of neurons, as they promote axonal growth, deliver essential neurotrophic factors, and control blood-brain barrier integrity. Through their surface receptors and transporters, they control neurotransmitter levels at the synaptic cleft and regulate synapse formation [10, 11]. Thus, astrocytes regulate glutamate homeostasis by two major CNS glutamate transporters, GLT-1/EAAT2 and GLAST/EAAT1, which are expressed almost exclusively by astrocytes in adult mammals. In addition, astrocyte dysfunction may be the reason for the observed decrease in neurotrophic factors [12, 13], as well as for the oxidative stress [14] and neuroinflammation [15].

These observations led many academic groups and biotech companies, including Kadimastem to the rationale that ALS could be treated by implantation of normal astrocytes or cells with similar astrocytic characteristics from an external source, to support or replace dysfunctional ALS astrocytes [16]. Following encouraging preclinical proof-of-concept studies with various cell-based therapies in ALS rodent models, some of the cell therapies were next evaluated in clinical trials in ALS patients.

2. Pathophysiology of astrocytes in amyotrophic lateral sclerosis disease

The pathological mechanisms for ALS are still not well understood and the proposed mechanisms include inflammation, oxidative stress, cytotoxicity by glutamate, and protein aggregation. Though MNs are the main affected cells in the disease, growing body of evidence suggests the involvement of astrocytes in the pathology of ALS in a noncell autonomous pathway. The contribution of astrocytes to the pathology of ALS is probably a combination of loss of homeostatic functions and/or gain of toxic functions.

2.1. Toxicity of astrocytes from ALS patients

Astrocytes that were isolated from sporadic and familial postmortem ALS patients and astrocytes derived from iPSC of ALS patients have been shown to be toxic to healthy (WT) motor neurons [17, 18]. Similar results were obtained by primary astrocytes isolated from hSOD1^{G93A} mouse model [19, 20]. The toxic effect of astrocytes on MNs was demonstrated also by addition of astrocyte condition medium [21, 22]. This lead to the notion that astrocytes of ALS patients secrete toxic/mutated proteins that cause specific death of MNs. This hypothesis is supported by data from in vivo studies in ALS models. Intraspinal transplantation in WT rats of mutated SOD1 astrocytes, but not WT astrocytes, led to deterioration of MNs. MN death

was suggested to be mediated by microglia activation since no activation of microglia was observed with WT astrocyte injection [23]. In addition, in ALS mouse models where SOD1 transgene was deleted specifically in astrocytes, the animals exhibited a delay in disease onset and slower progression [24–26]. Another study demonstrated that selective expression of mutant Tar DNA-binding protein 43 (TDP-43, found in ALS) in astrocytes causes a progressive loss of motor neurons and the denervation atrophy of skeletal muscles, resulting in progressive paralysis [27]. In addition, spinal cord astrocytes were found to degenerate in the microenvironment of motor neurons in hSOD1^{G93A} mouse model [28] and ubiquitin-positive inclusions were shown in MN microenvironment close to disease onset [29].

2.2. Failure in supporting MNs

Excessive stimulation of glutamate receptors causes excitotoxicity to neurons [30]. Reduction of functional astrocytic glutamate transporters is suggested to contribute to glutamate excitotoxicity found in ALS patients [31]. GLT-1, a glutamate transporter (a.k.a EAAT2) was found impaired in ALS patients [32, 33]. In vivo studies have demonstrated that focal loss of GLT-1 in the ventral horn of the spinal cord precedes disease onset in transgenic rat model for ALS overexpressing Cu(+2)/Zn(+2) superoxide dismutase 1 (SOD1) [34]. Transplantation of SOD1^{G93A} (glial-restricted precursor cells-glial progenitors that are capable to differentiate into astrocytes) in the cervical spinal cord of WT rats induced host MN ubiquitination and death, forelimb motor and respiratory dysfunction, reactive astrocytosis, as well as reduced GLT-1 transporter expression [23]. Mutating the caspase-3 cleavage consensus site in the GLT-1 sequence (D504N), inhibits caspase-3 deactivation of GLT-1. GLT-1^{D504N} mutation in SOD1-^{G93A} mice slowed down disease progression time, delayed the development of hindlimb and forelimb muscle weakness, and significantly increased the lifespan of the diseased mice [35]. Activation of mGlu3 metabotropic glutamate receptors in hSOD1^{G93A} mice enhances GLT-1 formation as well as secretion of glial-derived growth factor (GDNF) in the spinal cord and rescues motor neurons [36]. Several lines of evidence indicate that strategies designed to increase GLT-1 expression have a potential to prevent excitotoxicity; for example, the pyridazine derivative LDN/OSU-0212320 promotes GLT-1 translation [37], ceftriaxone increases GLT-1 expression by triggering NF-kB activity [38], and immunophilin ligand GPI-1046 also increases expression of GLT-1 [39]; all have been found to delay disease development and death of SOD1^{G93A} mice. However, a clinical trial evaluating ceftriaxone in ALS has been prematurely stopped because of lack of efficacy [40]. Nevertheless, Riluzole the first FDA-approved drug for ALS was found to increase glutamate uptake by C6 astroglial cells [41] shedding light on its therapeutic mechanism.

2.3. Cytotoxic cytokines

Inflammation-mediated neuronal injury is also recognized as a major factor to promote ALS disease progression and amplifies MN death-inducing processes. The neuroimmune activation is not only a physiological reaction to cell-autonomous death, but also an active component of nonautonomous cell death. Astrocytes participate in the cellular response to damage and danger signals by releasing inflammation-related molecules like NO, IL-6, INF-γ, prostaglandin D2, TGF-β, and TNF-α that can induce the apoptosis of neurons observed in ALS disease [21, 42–46]. INF-γ, for instance, was found to be expressed in GFAP-positive cells in the CNS [47] that possess a neurotoxic activity [48]. INF-γ was shown to induce selective death of motor neurons

through activation of lymphotoxin-β receptor via LIGHT. The ablation of LIGHT was shown to slow down disease progression and prolonged animals' life span [49, 50]. Cerebrospinal fluid-targeted delivery of neutralizing anti-IFNγ antibody delays motor decline in an ALS mouse model [51]. Other example of a key proinflammatory mediator is prostaglandin E2 (PGE2). High levels of PGE2 were found in postmortem brain tissue, cerebrospinal fluid, and serum from patients with sporadic ALS [52, 53]. PGE2 levels were also elevated in both the cerebral cortex and spinal cord of $SOD1^{G93A}$ mice [54]. Moreover, the expression of cyclooxygenase (COX)-2, a key enzyme in the synthesis of prostaglandins, is higher in the spinal cord of ALS patients and model mice [55]. In addition, the level of microsomal PGE synthase-1 (mPGES-1), the final regulatory enzyme for PGE2 production, is up-regulated in the motor neurons of G93A mice [56]. Recently, it was found that PGE2 induced an upregulation of the EP2 receptor in motor neuron-like NSC-34 cells and lumbar motor neurons of ALS model mice [57].

2.4. Necroptosis

Astrocytes from both ALS patients and animal models were also found to induce MN death by activation of necroptosis [58]. Necroptosis is a form of programmed necrosis that is independent from the activation of caspases and involves loss of the plasma membrane integrity. The receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and mixed lineage kinase domain-like (MLKL) have been identified as effector proteins of necroptosis. In vitro inhibition of the necroptosis pathway by the RIPK1 antagonist necrostatin-1 (Nec-1) or by direct silencing of RIPK1 via a short hairpin RNA (shRNA) has been reported to protect MNs from astrocyte-induced toxicity [59, 60]. Necrosulfonamide that inhibits MLKL was shown to almost completely rescue MNs from astroglial toxicity. The mechanism by which astrocytes induce necroptosis is still not understood. However, the factors TNF-α, TRAIL, and FasL were suggested to play a role in induction of necroptosis.

2.5. Mitochondrial alterations

Mitochondrial alterations have been observed in both neuronal and glial cells of ALS patients as well as in ALS animal models [61–63]. As mitochondria are both the main producers and target of reactive oxygen species (ROS), increased mitochondrial ROS production in ALS may lead to mitochondrial dysfunction and cell death. Mitochondrial dysfunction in $SOD1^{G93A}$-bearing astrocytes resulted in enhanced generation of reactive oxygen species (ROS) that promoted motor neuron degeneration [64, 65]. Mitochondria from rat $SOD1^{G93A}$-bearing astrocytes are defective in respiratory function and show an elevation in superoxide radical formation [64]. Thus, restoring mitochondrial dysfunction or reducing oxidative stress is an attractive therapeutic approach to treat ALS. For example, blocking the interaction of mutant SOD1 with one of its mitochondrial targets, Bcl-2, restores mitochondrial function in ALS mice [66]. In addition, Edaravone (a.k.a Radicava) that was recently approved by the FDA for the treatment of ALS possess a broad free radical scavenging activity and protects neurons, glia, and vascular endothelial cells against oxidative stress [67].

2.6. Neurotrophic factors

In both physiological and pathological conditions, astrocytes secrete a wide range of factors with multiple influences on their cellular neighbors. A well-known factor that is secreted by astrocytes is

the glial cell line-derived neurotrophic factor (GDNF), one of the most potent protective agents for motor neurons. Disruption of the astrocytic TNFR1-GDNF axis accelerates motor neuron degeneration and disease progression [68]. Astrocytes in ALS rat model acquire an accelerated senescent phenotype and show a reduced support in motor neurons, which can be partially reversed by glial cell line-derived neurotrophic factor (GDNF) [69]. Another factor that plays a role in ALS pathology is vascular endothelial growth factor (VEGF), originally described as a factor with a regulatory role in vascular growth and development, but which also directly affects neuronal cells [70, 71]. Transgenic mice expressing reduced levels of VEGF develop late-onset MN pathology, similar to that of ALS [72]. VEGF is secreted by astrocytes and has been shown to protect MNs from excitotoxic death, which occurs in ALS [72]. VEGF delays MN degeneration and increases survival in animal models of ALS [73, 74]. In line with these results, low levels of VEGF and GDNF were reported in the CSF of ALS patients [75]. VEGF exerts its antiexcitotoxic effects on MNs through mechanisms involving VEGF receptor-2 and activation of the PI3-K/Akt signaling pathway [72].

Thus, astrocytes play a pivotal role in the pathology of ALS and contribute to MN loss. A therapeutic approach would therefore be a replacement, or support, malfunctioning astrocytes in ALS with wild-type healthy astrocytes or modified cells with astrocytic characteristics. Such cells can mitigate the toxic CNS environment, modulate neuroinflammation, secrete neuroprotective factors, and foster MN repair process.

3. Cell sources for derivation of astrocytes

3.1. Glial restricted progenitors (GRP)-derived astrocytes

Glial restricted progenitors (GRP) are early cell population of the CNS that can self-renew and give rise to astrocytes and oligodendrocytes [76, 77]. Isolation of GRPs from human fetal tissues (i.e., 20-week-old fetal cadaveric brain tissue) [78] was described. In vitro studies demonstrated the capacity of these cells to differentiate toward astrocytes by using platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), T3 thyroid hormone, and ciliary growth factors (CNTF) as well as bone morphogenic proteins (BMPs) [78–81]. Yet, in vitro, only a subset of GRPs give rise to mature astrocytes [82]. In vivo transplantation of human GRPs into the spinal cord-injured animals demonstrated survival and differentiation toward astrocytes [83]. Moreover, intraspinal transplantation of GRPs overexpressing GLT-1 into ventral horn following cervical hemicontusion (injured spinal cord) significantly increased GLT1 protein expression and functional glutamate uptake following astrocyte differentiation. Transplantation into C4 hemicontusion compared to sham-injected animals demonstrated the paradigm that transplantation of GRPs might be a promising approach in cell therapy [79]. Yet, investigating human astrocyte maturation using a primary brain tissue obtained from cadaveric donors is challenging. Sample availability is limited, particularly for critical developmental time periods such as late gestation or early postnatal stages. In addition, derivation of homogenous astrocyte populations from GRPs is still a great challenge.

3.2. Derivation of astrocytes from embryonic stem cells or induced pluripotent stem cells

Two main pluripotent stem cells (PSC) sources for derivation of human astrocytes are embryonic stem cells and induced pluripotent stem cells (iPSCs), which are generated from adult human

somatic cells that are reprogrammed into pluripotent stem cells using multiple technologies [84]. Both cell sources possess astonishing capacity to undergo unlimited self-renewal and to differentiate into all cell types present in the adult body. These sources potentially provide unlimited supply of cells for cell-based therapy and drug screening platforms. Methods for producing neural precursor cells from PSC and their further differentiation toward glial lineage were demonstrated in pioneering studies in animal models of neurodevelopment [85–92]. In these studies, the key steps in neural commitment in vivo were identified and were recapitulated in a stepwise process of neural commitment in vitro that results in specific commitment of pluripotent stem cells toward neural and glial lineage. The differentiation process is usually done by exposing iPSC as well as hESC to different morphogens and mitogens [93] and specifying the different subtypes of neural and glial cells. Examples for such mitogens include sonic hedgehog (SHH) [94], which was found to be secreted in vivo from the notochord and neural tube, and WNT proteins [95] and bone morphogenetic protein (BMP) [96] that are secreted from the dorsal regions. This allowed specifying different subtypes of neural and glial cells. Other key factors, which are often used for the differentiation into neural progenitor fate, include fibroblast growth factors (FGFs), epidermal growth factors (EGFs), and retinoic acid (RA) [97, 98].

Recently, formation of organoids, a simplified version of an organ produced in vitro in three dimensions (3D), is being used as an alternative method for deriving glial cells from hPSC. This 3D structure allows spontaneous recapitulation of morphogenic and mitogenic features that occurs during neurodevelopment [99, 100]. This platform allows to study neural development and model various neurodegenerative diseases.

3.3. Direct conversion of somatic cells into astrocytes (iAstrocytes) or astrocyte-like cells

Direct cell-reprogramming principle that was applied for derivation of iPSC (i.e., by transduction of specified transcription factors or by using a defined chemical cocktail [84] are now being applied for a direct conversion of somatic cells into neural cells and astrocytes. Although rapid progress has been made in converting somatic cells into neural stem cells, neurons, and oligodendrocytes, direct reprogramming of somatic cells into astrocytes remains largely behind. Recently, Caiazzo et al. described for the first time conversion of mouse fibroblast into astrocytes (iAstrocytes), comparable to endogenous brain astrocytes. This was carried out by transducing the transcription factors NFIA, NFIB, and SOX9, and these factors were found to be involved in astroglial commitment and enabled direct conversion into astrocytes [101]. Another approach for direct conversion or reprogramming of mammalian fibroblasts into astrocytes is culturing the cells in the presence of a small molecules cocktail that includes histone deacetylase inhibitor VPA and GSK3β inhibitor CHIR99021, among other factors. TGFβ Inhibitor was found to be the critical factor in this cocktail [102].

3.4. Mesenchymal stem cells

Mesenchymal stem cells (MSC) are adult multipotent precursors derived from various adult tissues and differentiate in vivo or in vitro into osteocytes, chondrocytes, fibroblasts, and adipocytes [103]. Recently, it was reported by several groups that MSC can also adopt a neural fate in appropriate in vivo or in vitro experimental conditions [104]. Recently, several laboratories have managed to differentiate MSC into astrocytes-like cells; for example, addition of cAMP-elevating agents, forskolin and 3-isobutyl-1-methylxanthine (IBMX), resulted in the expression of neural

markers including β-tubulin III (Tuj-1), neuron-specific enolase (NSE), microtubule-associated protein-2 (MAP-2), nestin, and glial fibrillary acidic protein (GFAP) [105]. Another study showed that by using subsonic vibration (SSV) on MSC promoted their differentiation into neural-like cells in vitro [106]. Other studies developed protocols that induce adult human bone marrow-derived mesenchymal stem cells (MSCs) into becoming neurotrophic factor secreting astrocyte-like cells and attenuated clinical symptoms in animal model of multiple sclerosis and ALS [107–109].

4. Preclinical studies using cell-based therapies in ALS rodent models

4.1. Transgenic rodents overexpressing the mutant gene hSOD1G93A as a model for ALS

Several mutated genes have been identified as ALS causing mutations including C9ORF72, Cu/Zn superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TDP-43), UBQLN2, p62, VCP, Profilin1, and Matrin 3 [110]. Mutated SOD1 is the second most abundant ALS causing gene after gene encoding C9ORF72 and found in about 15% of the familial ALS cases [4, 111]. Several genetic mouse models expressing various ALS mutant genes were developed in order to mimic the human disease [7, 34, 112–115]; however, overexpression of the mutant SOD1 gene was shown to best recapitulate the pathology of the human disease [116]. Among SOD1 mutations, transgenic mice and rats overexpressing the human mutated gene $SOD1^{G93A}$ is the most used model of ALS in preclinical in vivo studies toward clinical trials [116]. Transgenic $hSOD1^{G93A}$ mice and rats exhibit histopathological hallmarks similar to those associated with ALS in humans with a massive degradation of motor neurons [7, 34]. The transgenic rodents show a clear disease pathology, including selective death of spinal cord motor neurons and muscle atrophy in both hind and forelimbs, early astrogliosis and microgliosis, glutamate-mediated excitotoxicity, protein aggregation, mitochondrial dysfunction, and impaired axonal transport [116–118]. Upon disease onset, $hSOD1^{G93A}$ mice progressively develop symptoms that include hyperreflexia and shaking of the limbs, decrease in locomotor activity, impairment in walking patterns, decreased grip strength, and impaired coordination. In the late stage of the disease, the mice develop a severe paralysis [7, 34].

4.2. Preclinical studies using human neural stem cells

Neural stem cells (NSC) are derived from CNS tissue at various developmental stages of embryogenesis, fetal and in adults. NSCs can potentially form all neural types of cells of the CNS including astrocytes, neurons, and oligodendrocytes [119]. The differentiation capacity of NSCs depends on the developmental stage from which the cells are isolated and might be wider when cells are isolated at early stage [120]. The NSC features of multipotency, homing, neurotrophism, immunomodulation, and neuroprotection make them a promising therapeutic candidate for ALS [121–126]. Several studies reported a beneficial effect on disease progression and survival of ALS rodent models following a direct injection of NSCs into the spinal cord parenchyma. Fetal spinal cord NSCs that were injected into the lumbar spinal cord of $SOD1^{G93A}$ rats completed their differentiation and secreted neurotrophic factors to the proximate areas. The transplanted cells also formed synaptic contacts with host motor neurons. Transplantation

of the cells delayed the disease onset, attenuated the progression, and moderately expanded life expectancy of the diseased rats by 10 days [127]. In order to support more muscle groups in SOD ^{G93A} rats, including muscles of hind- and forelimbs and respiratory, NSCs were injected into the ventral horn of both the lumbar and cervical spinal segments of presymptomatic animals. The dual treatment extended the survival of the rats by 17 days and delayed disease onset by 10 days compared to control animals, demonstrating the advantage of multiple injections [128]. A different study involving injection of NSCs to the lumbar ventral horn of presymptomatic SOD1 ^{G93A} rats did not demonstrate similar improvement in survival. However, the study still showed a limited and transient protection of motor function in the experimental animals [129]. The study has also demonstrated that the graft provided a neuroprotective effect, which was limited to the motor neurons of the lumbar segment. The grafted cells expressed markers of early mitotic neurons, including human neuron-specific enolase and doublecortin. In addition, the hNSCs reduced astrogliosis and numbers of activated microglia at the site of injection. Knippenberg et al. showed that intraspinal transplantation of human spinal cord-derived neural progenitor cells into the lumbar spinal cord of hSOD1^{G93A} mice delayed accumulation of motor deficiencies in a narrow time window during disease progression and moderately increased the life span by 5 days. Interestingly, female mice responded slightly better to the cell treatment, as observed in other studies in transgenic mutant hSOD1 mice [130, 131]. Characterization of the graft 6 weeks post-transplantation revealed that the cells were positive for the early neural marker nestin and rarely expressed the glial marker GFAP. Elevation of endogenous neurotrophic factors, but not human-derived factors, was measured in the spinal cord parenchyma [132]. A meta-analysis of intraspinal NSC transplantations in SOD1^{G93A} transgenic mice of 11 independent studies, performed by a consortium of ALS investigators, suggests that transplantation of NSCs from either human or mouse source delays disease onset, slows down symptom progression and prolongs survival of the mutated mice (e.g., the more than 1 year life extension in 25% of the mice that were injected at 4 sites). The authors proposed several mechanisms by which NSCs exert their therapeutic effects, including production of neurotrophic factors, preservation of motor function, and attenuation of inflammation and astrogliosis processes [133].

4.3. Preclinical studies using human mesenchymal stem cells

Mesenchymal stem cells (MSC) can be isolated from the placenta and from adult bone marrow and adipose tissues [134]. Although MSCs are of mesoderm origin, they maintain the potential to differentiate to cells types other than mesoderm derivatives including neurons-like cells and glial-like cells [135, 136].

MSC availability, well-established methods for harvesting and expansion, immunomodulatory features, ability to release neurotrophic factors, and lack of ethical issues make them attractive candidates for cell therapy applications [137, 138]. Several studies explored the therapeutic potential of MSCs in ALS models. These studies delivered MSCs to diseased animal through various routes, local and systemic, including intraspinal, intrathecal, intramuscular, and intravenous [137–140].

Transplantation of hMSCs into the lumbar spinal cord of asymptomatic SOD1^{G93A} mice reduced astrogliosis microglial reactivity and improved motor neuron counts. However, changes were gender dependent and observed only in female mice. On the other hand, behavioral tests demonstrated that the improvement in motor performance was restricted to transplanted males [141]. Boucherie et al. reported an alternative delivery approach of

rat MSCs by intrathecal injection to the cerebrospinal fluid of symptomatic hSOD1^{G93A} rats [142]. The transplantation led to infiltration of the injected cells into the CNS parenchyma including to the ventral horn. Transplantation of MSCs partially rescued motor neurons in the ventral horn, prolonged animal survival and improved motor performance over sham-injected rats [142].

A systemic administration of murine adipose-derived MSCs to hSOD1G93A mice by intravenous injection upon onset of disease symptom showed that a restricted number of labeled cells were able to reach the parenchyma of the spinal cord, with no evidence of neural differentiation. Upon transplantation, an increase in GDNF and bFGF levels was measured in the spinal cord. Researchers reported a better MN survival and a reduced reactive astrogliosis in the spinal cord in addition to amelioration of the course of disease progression [143]. A different study using an intravenous delivery of murine MSCs to SOD1G93A mice also prolonged survival and increased motor functions, in addition to improvement in histological pathology traits [144].

Another promising approach to rescue motor neurons in ALS is secretion of neurotrophic factors at the site of the damage by grafted cells. Human MSCs overexpressing the neurotrophic factor GDNF were injected into three muscle groups of presymptomatic SOD1G93A rats. The cells survived in the muscle and helped to preserve neuromuscular junction innervations. While engineered-MSC injection did not affect disease onset, it delayed disease progression and profoundly increased overall lifespan by up to 2 weeks [145]. Additional study demonstrated the advantage of combined delivery of hMSCs expressing both GDNF and VEGF. The NTF combination synergistically prolonged survival and attenuated disease progression in SOD1G93A rats [146].

4.4. Preclinical studies using human glial-restricted cells derived aborted human fetuses

Glial-restricted precursors (GRP) can be isolated from embryonic CNS tissue. These cells maintain a limited differentiation capacity to form only glial cells including astrocytes and oligodendrocytes [147, 148]. Endogenous glial cells in ALS experimental models and in patients were shown to be malfunctioning and even toxic to motor neurons, contributing to disease progression. Thus, introduction of healthy functional astrocytes to damaged areas in the CNS can potentially compensate for diseased astrocytes. Transplantation of rat GRPs into the cervical spinal cord of SOD1^{G93A} rats was found to maintain respiratory motor function. In this study, the cells robustly survived, migrated within the cervical spinal cord, and specifically localized in the ventral horn. At the time of transplantation, most of GRPs were nestin+, but they efficiently completed their differentiation into GFAP+ astrocytes by end-stage disease (87% GFAP+ astrocytes). At this stage, approximately only 10% of the cells remained as undifferentiated nestin + cells. During the course of disease, the transplanted astrocytes developed mature astrocyte morphologies and spatially interacted with host MNs in the spinal cord. GRP transplants slowed cervical spinal cord motor neuron loss and reduced microgliosis in the cervical segment. Overall, the cell transplantation extended animals' survival and attenuated declines in motor performance [149]. The authors tried to reproduce these results by the injection of human GRP to SOD1^{G93A} mouse model. However, although the cells could survive in the cervical spinal cord under intensive immune suppression regimen and differentiate into GFAP+ astrocytes, the graft did not protect motor neuron loss or motor function and did

not extent life expectancy. The difference between the outcomes of the two studies might be attributed to the different rodent models, cell dose, and number of injection sites [80].

4.5. Preclinical studies using human glial progenitors derived from ESCs and IPSCs

Embryonic stem cells are isolated from the inner-cell mass of a blastocyst and can be expanded in culture without losing their self-renewal capacity [150]. The cells can give rise to any cell type of the body. Induced pluripotent stem cells (iPSCs) are derived from somatic cells, mostly fibroblasts, which acquire an ESC-like pluripotent state after reprograming by induction of specific transcription factors. iPSCs can be generated from the patient's own cells. Transplantation of cells derived from such autologous iPSCs reduces the risk of immune rejection without the need of immunosuppression [151]. Kondo et al. differentiated human iPSCs into glial-rich neural progenitors (hiPSC-GRNP), highly enriched with GFAP+ cells. hiPSC-GRNPs were injected bilaterally into the lumbar spinal cord of transgenic $SOD1^{G93A}$ mice after disease onset. Treated mice showed an improvement in motor function and a prolonged survival of 12 days over sham-injected group. Transplanted cells survived in the spinal cord and differentiated mainly into $GFAP^+$, $ALDH1L1^+$, and $GLT\text{-}1^+$ astrocytes. Analysis of NTFs expression at the lumbar spinal cord reveled upregulation in mouse-originated VEFF, NT3, and GDNF [152].

We developed a protocol to produce large quantities of highly enriched astrocyte progenitors (APC; >90% GFAP+ cells) from human embryonic stem cells (hESC) according to GMP standards (unpublished data by the authors). In vitro, these cells express astrocyte markers including GFAP, S100β, GLAST, GLT-1, and Aquaporin- 4, and possess the activities of functional healthy astrocytes upon differentiation into mature astrocytes. These astrocytes are shown in vitro to have multiple activities including (1) protection of spinal cord motor neurons from oxidative stress produced by H_2O_2, (2) efficient glutamate uptake, which is in part due to GLT-1 (as shown by GLT-1 inhibitors), (3) stimulation of axonal growth in neurons seen in co-cultures with hES-AS, and (4) secretion of many factors with neuron protecting and stimulating activities. Intrathecal transplantation of hESC-derived APCs to the cerebrospinal fluid (CSF) of $SOD1^{G93A}$ transgenic rats and mice showed that the cells distribute along the neural axis and attach to the spinal cord and brain meninges, mainly to pia mater. In these studies, intrathecal transplantation of hESC-derived APCs significantly delayed disease onset and improved motor performance compared to sham-injected animals. The cells were shown to be safe and express markers of mature astrocytes including GFAP, GLAST, GLT-1, and Aquaporinn-4 in vivo. The cells did not express pluripotent markers and did not form teratomas or other tumors after a follow-up duration of 9 months. These cells are now the basis for a planned clinical trial.

5. Translation into the clinic

Following encouraging preclinical proof-of-concept studies with various cell-based therapies in ALS rodent models, demonstrating the safety and efficacy of the treatments, some of the cell therapies were already evaluated in clinical trials in ALS patients.

5.1. Route of cell administration

Several aspects of route of administration of cell therapies to ALS patients should be considered to ensure long-term survival, homing, and functionality of the cells in the target organ after transplantation. Cells for ALS treatment can be delivered by several routes, local or systemic, including intraspinal, intrathecal, intraventricular, intramuscular, and intravenous injections. Among these routes, intraspinal and intrathecal cell delivery routes were mostly used in ALS clinical studies [153, 154]. Intraspinal injection allows delivery of cells to the region of ventral horn in close proximity of motor neurons. However, migration of cells distal to injection site along the spinal cord is limited [80, 155] and therefore, only neural projections at the vicinity of the injected site are expected to be affected. Although intraspinal injection of cell was demonstrated to be a relatively safe procedure in animal models and in humans [156, 157], it is still a very challenging invasive procedure that requires an expertise and unique surgery instruments [158]. In addition, in order to support several groups of muscles in the patient's body, multiple independent injections along the spinal cord are required, increasing the complexity of the surgical procedure [159]. An alternative delivery of cells to the CNS is intrathecal injection to the subarachnoid space. Intrathecal injection is a routine procedure performed in humans by lumbar puncture. The intrathecal delivery is considered as a safe and simple method and does not require high level of expertise or instruments. IT injection allows the cell to distribute along the neuroaxis, distal from the injection site (unpublished data by the authors). Studies in animal models demonstrated a limited infiltration of engrafted cells from the CSF into the neural parenchyma [107, 160, 161]. Nevertheless, secreted factors such as NTFs, and anti-inflammatory/immunomodulatory cytokines circulate with the CSF and can diffuse into the parenchyma. In addition, the transplanted cells can remove from CSF circulating toxic factors such as excess glutamate and ROS. Therefore, the biodistribution of the cells in the CSF by IT injection is expected to exert systemic effect in the CNS, affecting both upper and lower MNs.

5.2. Clinical trials using cell-based therapies for the treatment of ALS

Cell therapy for ALS is considered as an innovative approach and many of the trials tested the cell therapy for the first time in humans. The primary endpoint of most studies was safety and the secondary endpoint included efficacy measurements. However, due to the small size studies and lack of placebo groups, the interpretation of the efficacy outcomes is difficult. Two, phase I and phase II, clinical trials (NCT01348451 and NCT 01730716) in ALS patients were conducted by Neuralstem Inc. [157, 162, 163]. The source of the human NSCs was a stem cell line generated from cervico-thoracic segments of spinal cord of a single 8-week-old aborted fetus. The cells were transplanted by an intraparenchymal injection procedure, performed using a spinal-mounted stabilization surgical device following laminectomy. Various concentrations of 0.5–16 × 10^6 cells were delivered to the lumbar and/or cervical vertebral levels. The primary endpoint of the two studies was safety. Adverse events were associated mainly with transient pain from the surgery procedure and to side effects of the immunosuppressive drugs. The efficacy of the treatment was evaluated by measuring ALSFRS-R, %, predicated forced vital capacity (FVC) and grip strength. Since the study did not include a randomized placebo group, the efficacy outcomes were compared to historical data. Although the efficacy data did not show an advantage of the treatment over historical controls, the small size groups, lack of placebo arms

and variability in disease progression between participants, make it difficult to draw a conclusion about the therapeutic benefits of the treatment. Graft survival was analyzed in six autopsy cases. Transplanted cells were identified in all cases by qPCR at the injection site in all cases, up to 2.5 years after cell injection. The presence of donor cells represented 0.67–5.4% of total tissue DNA. In one female patient, the injected cells, which were of male origin, were identified in histological sections 196 days post-transplantation by FISH targeting the Y chromosome. Some cells in the graft of this female patient completed their neural differentiation and expressed the neuronal marker NeuN, while other remained positive to the neural progenitor marker SOX2. However, many of the XY donor cells were negative to both markers with an unknown identity. These results demonstrate the survival of the graft in the patient under immune suppression. Yet, the interaction of the cells with the surrounding tissue and their effect of MNs in the spinal cord were not explored [164]. Another clinical trial using hNSCs for the treatment of ALS was conducted by Mazzini et al.. Under this trial, hNSCs were injected into the thoracic spinal cord segment of ALS patients (EudraCT:2009–014484-39). The NSCs were isolated from the forebrain of aborted fetuses and expanded in culture under GMP conditions. Upon laminectomy, 2.25–5.5 × 10^6 cells were injected unilaterally or bilaterally into the T8-T11 ventral horn of six ALS patients. No severe adverse events were related to the treatment, and the most common reported adverse event was transient postsurgical pain. Patients were monitored for 1 year on a monthly basis and then for every 3 months. Clinical assessments up to 18 months after transplantation showed no acceleration in the disease progression that could be related to the treatment. A transitory improvement of the ambulation abilities was reported in two patients and one patient demonstrated a transient improvement in muscle power of lower limbs [156].

The safety and efficacy of MSC transplantation for treating ALS was conducted by Mazzini et al. In total, 10 ALS patients were injected intraspinally at T4-T6 with 11–120 × 10^6 autologous MSCs (Italian registration number: 16,454-pre21–823). The cells were isolated from bone marrow and expanded ex vivo under GMP conditions. The patients have been monitored for at least a 24-month follow-up period after transplantation. No serious treatment-related adverse events were reported. Overall, the procedure was demonstrated to be safe with only transient adverse events that were associated with the surgery procedure. Yet, no significant changes in the progression of the disease were reported in the follow-up period [165]. In separate long-term consecutive phase I studies, 19 patients were followed for up to 9 years after intraspinal transplantation of autologous MSCs. The procedure was demonstrated to be safe and did not accelerate the progression of the disease. MRI analysis showed no structural changes from baseline and lack of tumor formation. However, no clinical benefits were observed in the patients during the follow-up phase [153, 166].

In order to improve the potential of MSCs to support motor neurons, Brainstorm cell therapeutics Inc. developed an in vitro procedure to expand autologous MSCs and induced them to secrete neurotrophic factors including GDNF, BDNF, VEGF and HGF. These NTF secreting cells were delivered to the cerebrospinal fluid by intrathecal administration and/or to motor end-plates by intramuscular (IM) administration [167].

The company conducted Phase I/II clinical trials in 26 ALS patients (NCT01051882 and NCT01777646). One million cells/sites at 24–48 separate sites were injected to the biceps and triceps, and 1–2 × 10^6 cells/kg were injected intrathecally. Overall, the treatment was found to be safe and tolerable by patients with only transient and mild adverse events appearing

after the administration of cells. The authors also reported an improvement in the decline of ALSFRS-R within 6 month of follow-up period compared to the run-in period, from −1.2 to 0.6 points/month, and also a decline from −5.1% to −1.2%/month in the predicted forced vital capacity [168]. The safety and efficacy of MSC-NTF cells were further tested in a randomized, double-blinded phase IIb clinical trial in 48 ALS patients, divided in a ratio of 3:1 between treatment and placebo arms, respectively (NCT02017912). According to the sponsor's website, the cells were injected both intramuscularly (48×10^6 cells at 24 sites) and intrathecally (125×10^6 cells), and patients were monitored for 24 weeks. Treatment was shown to be safe and well tolerated. A responder in the analysis of the trial was defined as a subject that who improved post-treatment compared with pretreatment run-in period. Data analysis demonstrated higher percentage of responders in the treatment arm subjects over placebo in most time points of the analysis. The responder analysis also revealed a subgroup of more rapidly progressing patients that were more likely to benefit from the treatment. The sponsor reported that the concentration of neurotrophic factors in the CSF including VEGF, HGF, and LIF elevated in the cell-treated arm after transplantation, but not in the placebo arm. These results were observed in parallel with a reduction in inflammatory markers in the CSF of cell-treated patients. According to the company's announcement, the efficacy of the therapy will be evaluated under a prospective placebo-controlled, multidose phase III trial in approximately 200 rapidly progressing ALS patients (NCT03280056).

Administration of autologous bone-marrow-derived MSCs by intrathecal injection to ALS patients was conducted by Corestem Inc., in a two-stage phase I/II clinical trial (NCT01363401). In the first stage of the study, cultured MSC expressing the markers CD29, CD44, CD73, and CD105 were administrated to the seven patients by two repeated LP injections (1 × 106 cells/kg), one monte apart. The patients were monitored for a period of 12 months. During the follow-up period, the treatment was shown to be tolerable and generally safe. Although the first stage of the study did not include a control group and was not powered to detect meaningful efficacy changes, the study showed encouraging results of stabilization of the ALSFRS-R score in all patients over a period of 6 months after first cell injection. In addition, levels of the immune response cytokines IL-10, TGF-β, and IL-6 were increased in the CSF after MSC injection, suggesting that of the effect of MSC injection on ALS patients is mediated, at least partially, by an immune response [169].

6. Conclusion

ALS is a multifactorial disease involving both genetic mutations and dysregulation of molecular pathways. Several mechanisms were identified in the pathophysiology of the disease, among them, glutamate excitoxicity, accumulation of free radicals, protein aggregation, mitochondrial dysfunction and impaired axonal transport and inflammation. In addition, during the last few years, growing evidence shows that astrocytes of both ALS animal models and ALS patients are malfunctional and even toxic. These astrocytes cannot support MNs and therefore contribute to the progression of the disease. Transplantation of healthy functional cells that can replace diseased astrocytes is, therefore, a promising strategy to treat ALS. Although different types of cells were proposed as a therapy for ALS, they all share mechanisms of action including, anti-inflammation/immunomodulation, clearing of toxic environment, and secretion of

neurotrophic factors. The combined mechanism of action provided by cell transplantation is postulated to better cope with the multifactorial nature of the disease compared to a single pathway-based drug. Preclinical studies in ALS animal models showed the high safety profile of cell-based treatments in addition to their benefits in delaying disease onset, slowing down clinical symptoms and in many cases also to extend survival. Besides behavioral measurements, many of the studies also demonstrated graft survival, decline in inflammation, and improvement in histopathological attributes of the disease. Translation of the preclinical studies into clinical trials confirmed the safety of the procedures. Efficacy in most of these trials was a secondary endpoint, and some studies showed moderate and/or transient beneficial effects. Yet, since most of the clinical trials were at early stage, with small-size groups without a control arm, it is difficult to evaluate the efficacy of the treatments. Late-stage, placebo-controlled clinical studies with greater number of patients will prove whether any of the cell-based therapies indeed change the course of the disease.

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